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(54) Method for enhancing enzyme activity at elevated temperature

(57) A method for enhancing activity of enzyme at an elevated temperature which comprises adding a substance exhibiting chaperone function such as a saccharide to a reaction mixture containing the enzyme. The method can improve activity of enzymes more easily and more effectively and hence afford increased enzyme activity at an elevated temperature.

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Description

BACKGROUND OF THE INVENTION

The present invention relates to a method for enhancing enzyme activity at an elevated temperature by using a substance exhibiting chaperone function.

In general, enzymes exhibit lower activity at a temperature above their optimum temperature than the activity at their optimum temperature. It is also known that their activity is lost when they are exposed to a temperature higher than a certain level. Depending on the kind of enzyme, a temperature at which such heat inactivation occurs may vary. However, most of enzymes having optimum temperature of ordinary temperature are inactivated when heated to around 50 °C. Enzymes stable at an elevated temperature are also known and such heat-resistant enzymes generally have a higher optimum temperature.

Depending on the conditions where enzymes are used, it is often desirable to use enzymes at an elevated temperature. In such a case, a heat-resistant enzyme as mentioned above is generally used. Examples of such a heat-resistant enzyme include Taq polymerase, which is frequently used for PCR. However, in many cases, a suitable heat-resistant enzyme may not be known, or even if a possible heat-resistant enzyme is known, other conditions to be used may not meet the enzyme.

For example, Superscript II is known as a reverse transcriptase (RNA-dependent DNA polymerase) which can afford a cDNA from a mRNA *in vitro*. Superscript II is a heat-labile enzyme exhibiting an optimum temperature of 42°C and completely inactivated at a temperature above 50°C within 10 minutes. Although Tth DNA polymerase is an enzyme having heat resistance and reverse transcription activity, it requires manganese ions for exerting the enzyme activity. If cDNAs are produced from mRNAs at a higher temperature using the Tth DNA polymerase, mRNAs are fragmented by manganese ions presented in a reaction system and therefore it becomes difficult to obtain full length cDNAs.

Magnesium ions required by a heat-labile reverse transcriptase such as Superscript II mentioned above may also cause the fragmentation of mRNA in a certain buffer or water at an elevated temperature. According to the present inventor's researches, manganese ions exhibit stronger fragmentation activity than magnesium ions and control of the fragmentation due to manganese ions is difficult even using a chelating agent.

Taq polymerase is known as an inherently heat-resistant enzyme. However, it shows reduction of activity during 25 to 30 cycles or more generally used in PCR. Therefore, if the reduction of Taq polymerase activity can be prevented, higher amplification effect and higher cycle number can be realized with fewer units of the enzyme.

In some cases, reverse transcription may be required to be performed at a temperature above 50°C for some reasons. For example, in order to obtain full length cDNAs, it is desirable to preform reverse transcription while preventing the formation of secondary structure of mRNAs.

However, a heat-resistant enzyme having reverse transcription activity such as Tth DNA polymerase cannot afford full length cDNAs. Therefore, it is necessary to utilize a currently available reverse transcriptase which is used at an ambient temperature.

Similar situation may be frequently found in other enzymes not only polymerases but also restriction enzymes .

For some enzymes, it has been known that an enzyme exhibiting a higher optimum temperature can be obtained by introducing a mutation through genetic engineering. However, such improvement of heat-resistance is not always possible and has not been known so long as reverse transcriptase concerns.

If an enzyme can exhibit higher activity at a higher temperature, its utility may be enhanced even though it is known as a heat resistant enzyme.

Therefore, the object of the present invention is to provide a method for easily and efficiently improving heat resistance of enzyme to obtain high enzyme activity at an elevated temperature.

DESCRIPTION OF THE INVENTION

The present invention provides a method for enhancing activity of enzyme which comprises adding a substance exhibiting chaperone function to a reaction mixture containing the enzyme.

Fig. 1 is a photograph showing the results of agarose gel electrophoresis obtained in Example 1.

Fig. 2 is a photograph showing the results of agarose gel electrophoresis obtained in Example 4 in which betain was used.

Fig. 3 is a photograph showing the results of agarose gel electrophoresis obtained in Example 4 in which sarcosine was used.

Fig. 4 presents relative activity of *Sty* I tested in Example 4 in which betain was used.

Fig. 5 presents relative activity of *Sty* I tested in Example 4 in which sarcosine was used.

In the method of the present invention, the objective enzyme is not particularly limited and may be an enzyme which is not inactivated and exhibits its activity at an elevated temperature. It may be possible to enhance an activity of enzyme at a higher temperature by applying the method of the present invention to an enzyme which is not permanently inactivated but exhibit substantially no activity or which is inactivated at an elevated temperature under ordinary conditions, so long as they are in a condition where activation at an elevated temperature is possible.

Typical examples of the enzyme to which the method of the present invention is applicable include

polymerases and restriction enzymes. Examples of polymerases include DNA polymerases, RNA-dependent DNA polymerases (reverse transcriptases), DNA replicases, terminal deoxytransferases, poly A polymerases and telomerases. However, the enzyme is not limited to these.

Examples of the DNA polymerase include Sequencease Ver.2, T7 DNA polymerase, T4 DNA polymerase, DNA polymerase I and the like. Examples of the heat-resistant DNA polymerase include Taq polymerase, Vent DNA polymerase, pfu polymerase, Tth polymerase, Thermosequenes and the like. Heat resistance of these heat resistant DNA polymerases can be further enhanced by the method of the present invention and therefore amplification ratio and cycle number of PCR can be increased to improve stability of PCR.

Examples of the RNA-dependent DNA polymerase (reverse transcriptase) include Seperscript II, AMV reverse transcriptase, MulV reverse transcriptase and the like.

In addition to such polymerases as mentioned above, some restriction enzymes such as Taq I are not inactivated and exhibit substantial activity at an elevated temperature. Such enzymes may also be stabilized at an elevated temperature by the method of the present invention. Examples of restriction enzymes to which the method of the present invention is applicable include *Sty* I, *Eco* RI, *Mlu* I, *Nco* I, DNase I, Rnase I, *Nde* I, *Pvu* II, *Pst* I, *Dra* I, *Hin* DIII and *Hin* cII. However, the enzyme is not limited to these.

In the method of the present invention, a substance exhibiting chaperone function is presented in a reaction mixture.

Examples of the substance exhibiting chaperone function include saccharides, amino acids, polyalcohols and their derivatives, and chaperone proteins. However, the substance is not limited to these. The "chaperone function" means a function for renaturing proteins denatured by stress such as heat shock, or a function for preventing complete denaturation of proteins by heat to maintain the native structure.

Examples of the saccharide exhibiting the chaperone function include oligosaccharides and monosaccharides such as trehalose, maltose, glucose, sucrose, lactose, xylobiose, agarobiose, cellobiose, levanbiose, quitobiose, 2-β-glucuronosylglucuronic acid, allose, altrose, galactose, gulose, idose, mannose, talose, sorbitol, levulose, xylitol and arabitol. However, the saccharide is not limited to these. Those saccharides mentioned above can be used alone or in any combination thereof. Among these, trehalose, sorbitol, xylitol, levulose and arabitol exhibit strong chaperone function and marked effect for activating enzymes at an elevated temperature.

Examples of the amino acids and derivatives thereof include N^{ε}-acetyl-β-lysine, alanine, γ-aminobutyric acid, betain, N^{α}-carbamoyl-L-glutamine 1-amide, choline, dimethylthetine, ecotine (1,4,5,6-tetrahydro-2-methyl-4-pirymidine carboxilic acid), glutamate, β-glutammine, glycine, octopine, proline, sarcosine, taurine and trymethylamine N-oxide (TMAO). However, the amino acids and derivatives thereof are not limited to these. Those amino acids mentioned above can be used alone or in any combination thereof. Among these, betain and sarcosine exhibit strong chaperone function and marked effect for activating enzymes at an elevated temperature.

The substance exhibiting chaperone function include polyalcohols. The saccharides are included in polyalcohols and other examples of the polyalcohols include glycerol, ethylene glycol, polyethylene glycol and the like. Those polyalcohols can be used alone or in any combination thereof.

The substance exhibiting chaperone function include chaperone proteins. Examples of the chaperone proteins include chaperone proteins of Thermophiric bacteria and heat shock proteins such as HSP 90, HSP 70 and HSP 60. Those chaperone proteins can be used alone or in any combination thereof.

These substances exhibiting chaperone function show different optimum concentrations for stabilizing the enzyme depending on the kind of the enzyme and the optimum concentration may vary among the substances for the same enzyme. Therefore, a concentration of particular substance to be added to a specific reaction system may be suitably decided depending on the kinds of the substance and the enzyme such as reverse transcriptase.

To enhance the effect of the substances exhibiting chaperone function such as saccharides, amino acids or chaperone proteins, one or more kinds of polyalcohols may be used in addition to one ore more kinds of the above substances. Examples of the polyalcohol include glycerol, ethylene glycol, polyethylene glycol and the like.

According to the method of the present invention, activity of enzyme such as a polymerase or a restriction enzyme can be enhanced at an elevated temperature. The term "elevated temperature" herein used refers to, for example, a temperature of 45 to 110°C. However, the temperature at which an enzyme can be stabilized may be vary depending on the kind of the enzyme. An enzyme usually used at an ordinary temperature may be stabilized at an elevated temperature higher than ordinary temperature, and a heat-resistant enzyme can be stabilized at a further elevated temperature higher than its optimum temperature.

According to the method of the present invention, not only heat-resistance of enzymes such as polymerases and restriction enzymes can be improved, but also activity of enzymes such as polymerases and restriction enzymes at an elevated temperature can be enhanced by using the above-mentioned substance exhibiting chaperone function.

EXAMPLES

The present invention will be further explained in detail with reference to the following examples.

Example 1

Improvement of reverse transcription efficiency by making reverse transcriptase heat-resistant

To examine reverse transcription activity of Superscript II at an elevated temperature, cDNAs were synthesized using RNAs transcribed *in vitro* by T7 RNA polymerase as template and the products were evaluated. By using RNAs as a template transcribed *in vitro* and evaluating the products by electrophoresis, reverse transcription efficiencies of the samples can be compared with one another and thereby non-specific transcription termination which leads to premature termination of reverse transcription and/or reduction of reaction efficiency can be evaluated. The template RNAs were prepared by transcribing pBluescript II SK, which had been cleaved into a linear form with a restriction enzyme *Not*I, with T7 RNA polymerase *in vitro*. This reaction was initiated from T7 promoter described in the instruction of pBluescript II SK.

As a control, the following standard buffer condition was used: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, each 0.75 mM of dNTPs (dATP, dGTP, dCTP and dTTP).

In the above standard buffer condition, 1 μg of template RNA, 400 ng of primer (20mer SK primer, CGCTCTAGAACTAGTGGATC) and 200 units of Superscript II were prepared and the final volume was adjusted to 20 μl. 0.2 μl of [α-^{32}P]dGTP was used for labeling of reverse transcription products. The RNA and the primer were incubated at 65°C before the other substrates were added. Then, the reaction was performed at 42°C for 1 hour. The reaction products were subjected to denatured agarose electrophoresis and electrophoretic patterns were examined by autoradiography to evaluate recoveries of full length cDNAs and rates of short products obtained from incomplete elongation. The results are shown in Lane 1 of Fig. 1.

The reverse transcriptase Superscript II was inactivated at a temperature of 50°C in the above standard buffer condition.

The following buffer condition for reverse transcription was used to verify that addition of oligosaccharide stabilizes the enzyme reaction: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, each 0.75 mM of dNTPs (dATP, dGTP, dCTP, dTTP), 20% (w/v) trehalose and 20% (v/v) glycerol.

1 μg of template RNA, 400 ng of primer (20mer SK primer) and 200 units of Superscript II were reacted in 24 μl of aqueous solution under the above buffer condition. 0.2 μl of [α-^{32}P]dGTP was used for labeling of reverse transcription products. Under this condition, the reverse transcriptase Superscript II exhibited higher activity than the control reaction at a normal temperature (42°C). The primer and the template RNAs were annealed at 37°C for 2 minutes and the enzyme activity was measured at 60°C.

The reaction products were subjected to denaturing agarose electrophoresis as described above, and electrophoretic patterns were examined by autoradiography to evaluate recoveries of full length cDNAs and rates of short products obtained from incomplete elongation. The results are shown in Fig. 1.

As shown in Lane 1, products resulted from premature termination of reverse transcription at specific sites or non-specific termination of reverse transcription were seen under the standard buffer condition at 42°C.

As shown in Lane 2, at 42°C as in Lane 1, such products resulted from premature termination as mentioned above were also observed even though 20% trehalose and 20% glycerol were added.

As shown in Lane 3, when the temperature was raised to 60 °C, the amount of products obtained from prematurely terminated synthesis became very small and full length products were synthesized.

As shown in Lane 5, when 0.125 μg/μl of BSA was added to the condition of Lane 3, the enzyme activity was further stabilized. However, BSA alone without 20% trehalose and 20% glycerol did not make the enzyme sufficiently heat-resistant.

As shown in Lane 4, when 0.05% of Triton X100 was added to the condition of Lane 3, the amount of incomplete reverse transcription products was further reduced. However, the whole activity of the reverse transcriptase was slightly reduced.

Example 2

Reaction was performed under the same condition as Lane 3 of Example 1 except that glucose or maltose was used instead of trehalose. The electrophoretic pattern showed again that the amount of products obtained from prematurely terminated synthesis became very small and full length products were synthesized as in Lane 3 of Example 1.

Example 3

Reaction was performed under the same condition as Lane 3 of Example 1 except that arabitol, sorbitol, levulose, xylitol or betain was used instead of trehalose. The electrophoretic pattern showed again that the amount of products obtained from prematurely terminated synthesis became very small and full length products were synthesized as in Lane 3 of Example 1.

Example 4

Reaction solutions (20 μl each) containing a restriction enzyme, *Sty* I o.5 units, its substrate, λ DNA 0.5 μg

and betain 0-0.6M or sarcosine 0-3.6M were incubated at 37, 45, 50, 55 or 60°C for 1 hour. In order to prevent initiation of enzyme reaction before the incubation, the samples were quickly prepared on ice. Upon incubation, 0.25% bromophenol blue, 0.26% XC (xylene cyanol), 30% glycerol and 120mM EDTA (4 μl) was added to the reaction solution to terminate the reaction. The resulting solution was heated at 65°C for 5 minutes to melt cos sites and subjected to electrophoresis using 0.8% agarose gel containing 0.05% EtBr. A photograph showing the results of agarose gel electrophoresis regarding the samples using betin is presented in Fig. 2. A photograph showing the results of agarose gel electrophoresis regarding the samples using sarcosine is presented in Fig. 3.

After electrophoresis, image analysis of the gels was conducted. Enzyme activity was represented by comparing strength of bands appeared at 1kbp (with arrow). Regarding the samples using betain, the standard (100) is the band strength obtained from the sample using 0M of betain and incubated at 37°C. Relative activity of *Sty* I tested in the presence of betain is presented in Fig. 4. Regarding the samples using sarcosine, the standard (100) is the band strength obtained from the sample using 0M of sarcosine and incubated at 37°C. Relative activity of *Sty* I tested in the presence of sarcosine is presented in Fig. 5.

From the results, it can be seen that a restriction enzyme was thermally activatd by the addition of suitable concentration of betain or sarconsine.

Claims

1. A method for enhancing activity of enzyme at an elevated temperature which comprises adding a substance exhibiting chaperone function to a reaction mixture containing the enzyme.

2. The method of claim 1, wherein the reaction mixture is at a temperature of 45 to 110°C.

3. The method of claim 1 or 2, wherein the substance exhibiting chaperone function is one or more substances selected from the group consisting of saccharides, polyalcohols, amino acids and their derivatives, and chaperone proteins.

4. The method of claim 3, wherein the saccharide is one or more saccharides selected from the group consisting of trehalose, maltose, glucose, sucrose, lactose, xylobiose, agarobiose, cellobiose, levanbiose, quitobiose, 2-β-glucuronosylglucuronic acid, allose, altrose, galactose, gulose, idose, mannose, talose, sorbitol, levulose, xylitol and arabitol.

5. The method of claim 4, wherein the saccharide is trehalose. sorbitol, levulose, xylitol or arabitol.

6. The method of claim 3, wherein the amino acid or their derivative is one or more members selected from the group consisting of N^{ε}-acetyl-β-lysine, alanine, γ-aminobutyric acid, betain, N^{α}-carbamoyl-L-glutamine 1-amide, choline, dimethylthetine, ecotine, glutamate, β-glutammine, glycine, octopine, proline, sarcosine, taurine and trymethylamine N-oxide.

7. The method of claim 6, wherein the amino acid or their derivative is betain or sarconsine.

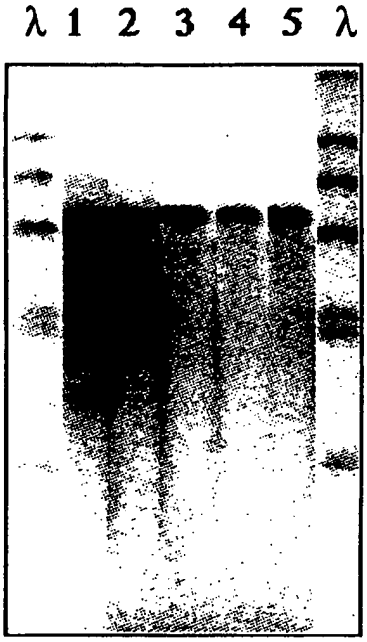
8. The method of claim 3, wherein the chaperone protein is selected from those of Thermophiric bacteria and heat shock proteins.

9. The method of any one of claims 1 to 8, wherein one or more polyalcohols are added to the reaction mixture.

10. The method of any one of claims 1 to 9, wherein the enzyme is a polymerase or a restriction enzyme.

11. The method of claim 10, wherein the polymerase is a reverse transcriptase or DNA polymerase.

Fig. 1

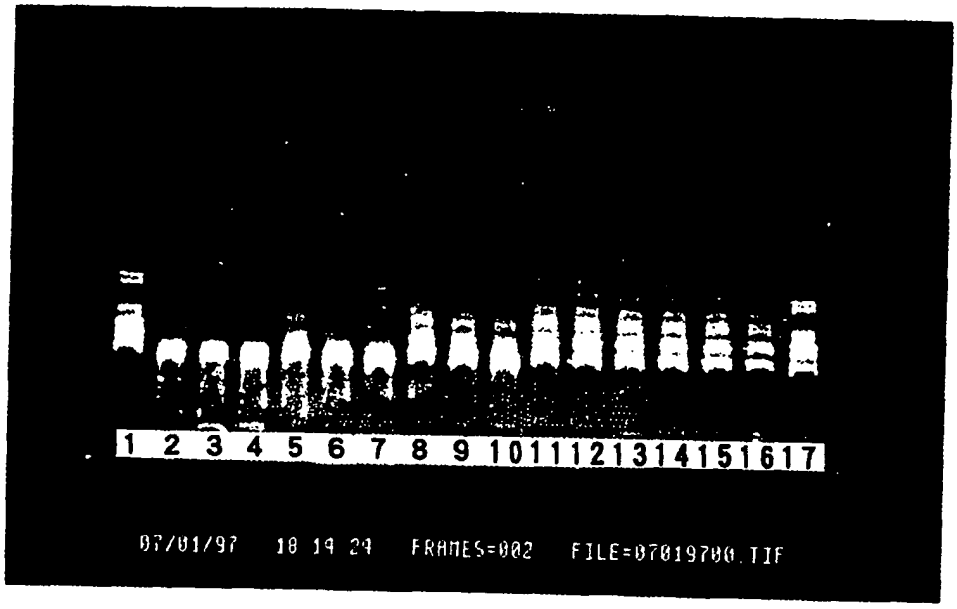


λ=Lambda HinDIII marker

1) Standard "optimized" buffer condition: Reaction temperature 42℃
2) Buffer containing trehalose (20%) and glycerol (20%): Reaction temperature 42℃
3) Buffer containing trehalose (20%) and glycerol (20%): Reaction temperature 60℃
4) Buffer containing trehalose (20%), glycerol (20%) and Triton X-100 (0.05%): Reaction temperature 60℃
5) Buffer containing trehalose (20%), glycerol (20%) and BSA (125 ng/μl): Reaction temperature 60℃

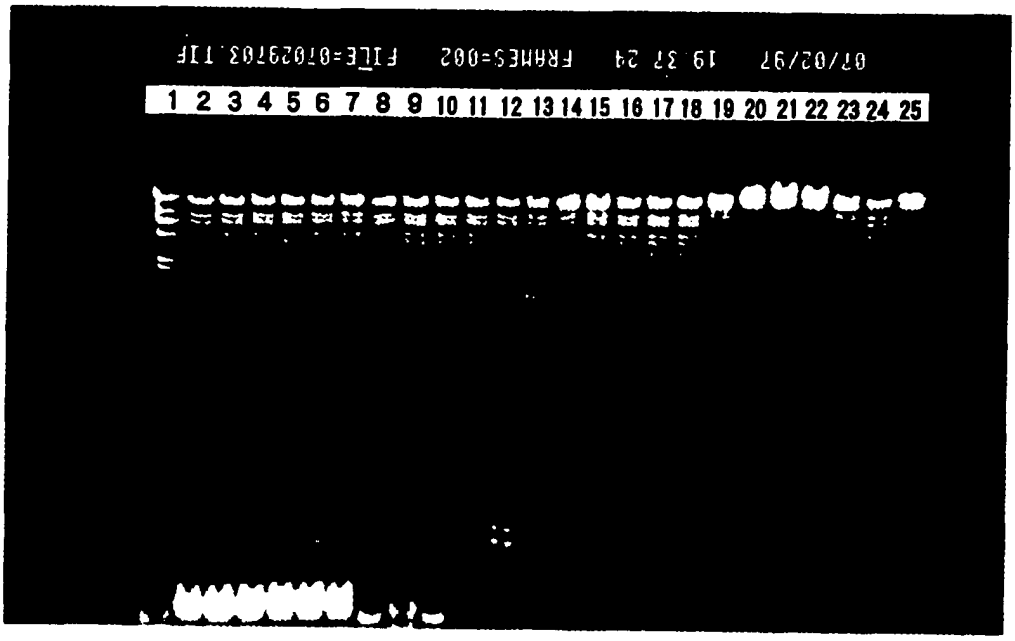
Superscript II is inactivated at a temperature of 50℃ or more under the standard buffer condition (not shown).

Fig. 2



1) 17) λ Hindlll
2) 37℃ 0 M
3) 37℃ 0. 2M
4) 37℃ 0. 6M
5) 45℃ 0 M
6) 45℃ 0. 2M
7) 45℃ 0. 6M
8) 50℃ 0 M
9) 50℃ 0. 2M
10) 50℃ 0. 6M
11) 55℃ 0 M
12) 55℃ 0. 2M
13) 55℃ 0. 6M
14) 60℃ 0 M
15) 60℃ 0. 2M
16) 60℃ 0. 6M

Fig. 3



1) λHindIII
2) 37℃ 0 M
3) 37℃ 0.2M
4) 37℃ 0.6M
5) 37℃ 1.2M
6) 37℃ 2.4M
7) 37℃ 3.6M
8) 45℃ 0 M
9) 45℃ 0.2M
10) 45℃ 0.6M
11) 45℃ 1.2M
12) 45℃ 2.4M
13) 45℃ 3.6M
14) 50℃ 0 M
15) 50℃ 0.2M
16) 50℃ 0.6M
17) 50℃ 1.2M
18) 50℃ 2.4M
19) 50℃ 3.6M
20) 55℃ 0 M
21) 55℃ 0.2M
22) 55℃ 0.6M
23) 55℃ 1.2M
24) 55℃ 2.4M
25) 55℃ 3.6M

Fig. 4

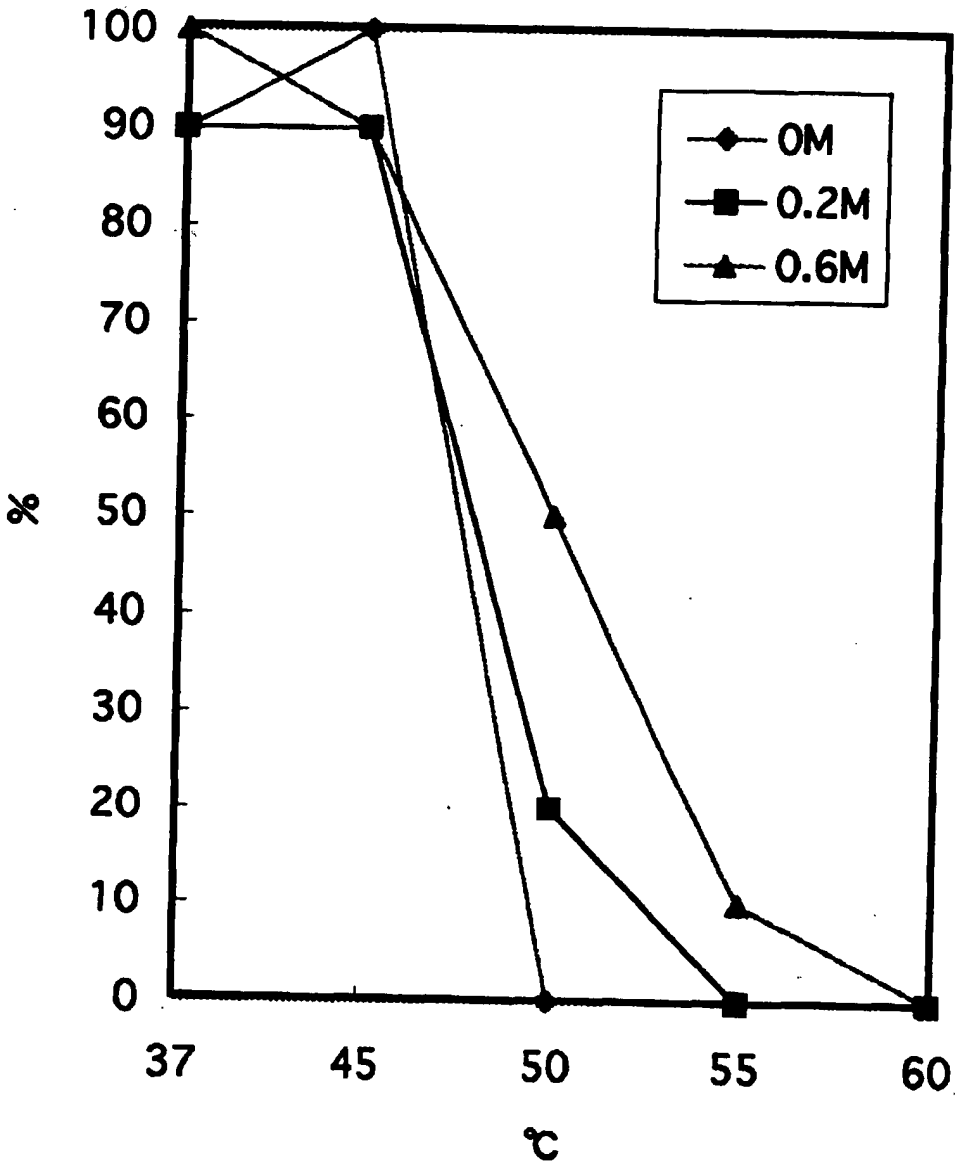


Fig. 5

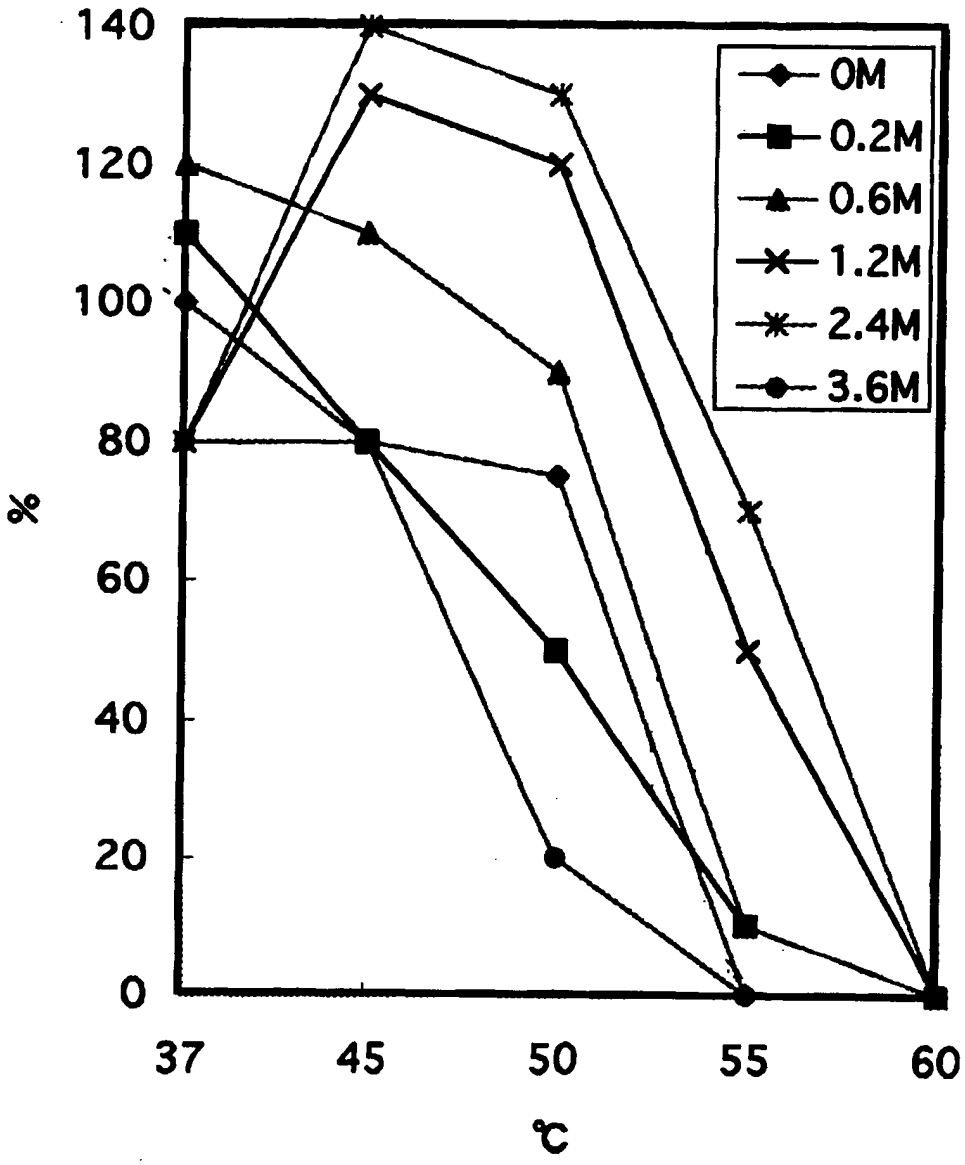


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Fig. 1

λ=Lambda HinDIII marker
1) Standard "optimized" buffer condition: Reaction temperature 42°C
2) Buffer containing trehalose (20%) and glycerol (20%): Reaction temperature 42°C
3) Buffer containing trehalose (20%) and glycerol (20%): Reaction temperature 60°C
4) Buffer containing trehalose (20%), glycerol (20%) and Triton X-100 (0.05%): Reaction temperature 60°C
5) Buffer containing trehalose (20%), glycerol (20%) and BSA (125 ng/μl): Reaction temperature 60°C

Superscript II is inactivated at a temperature of 50°C or more under the standard buffer condition (not shown).

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EUROPEAN SEARCH REPORT

Application Number
EP 97 11 2671

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X	JOURNAL OF BIOTECHNOLOGY., vol.7, no.4, 1988, AMSTERDAM NL pages 293 - 298, XP002054414 BERNIER F ET AL. 'STABILIZATION OF BETA-GALACTOSIDASE BY POLYHYDRIC ALCOHOLS' * the whole document * ---	1-5	
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X	WO-A-93 16175 (GIST BROCADES NV) * page 2, line 32 - page 3, line 12 * * page 6, line 11 - line 30 * ---	1,3-5,9	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N C12Q
X	EP-A-0 035 204 (CUTTER LABORATORIES) * page 2, line 15 - line 22 * * page 6, line 1 - line 15 * * page 11, line 1 - line 18 * ---	1-5	
A	EP-A-0 117 064 (THE GREEN CROSS CORPORATION) * page 3, line 2 - page 4, line 4 * --- -/--	1-5	

~~The present search report has been drawn up for all claims~~

Place of search	Date of completion of the search	Examiner
BERLIN	23 February 1998	De Kok, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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A	PATENT ABSTRACTS OF JAPAN vol. 095, no. 011 26 December 1995 & JP-A-07 194 378 (FUJI SEITO KK) 1 August 1995 * abstract *	1,3-5,10	
A	WO-A-96 15235 (ASAHI CHEMICAL IND) * abstract *	1,3-5, 10,11	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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